SETAC PRESS

Environmental Toxicology and Chemistry, Vol. 29, No. 1, pp. 220–229, 2010

Printed in the USA
DOI: 10.1002/etc.7

TOWARD A UNIFIED APPROACH TO DOSE–RESPONSE MODELING IN ECOTOXICOLOGY

CHRISTIAN RITZ*

Statistics Group, Department of Basic Sciences and Environment, Faculty of Life Sciences, University of Copenhagen, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark

(*Submitted* 16 *December* 2008; *Returned for Revision* 7 *May* 2009; *Accepted* 18 *June* 2009)

Abstract—This study reviews dose–response models that are used in ecotoxicology. The focus lies on clarification of differences and similarities between models, and as a side effect, their different guises in ecotoxicology are unravelled. A look at frequently used dose–response models reveals major discrepancies, among other things in naming conventions. Therefore, there is a need for a unified view on dose–response modeling in order to improve the understanding of it and to facilitate communication and comparison of findings across studies, thus realizing its full potential. This study attempts to establish a general framework that encompasses most dose–response models that are of interest to ecotoxicologists in practice. The framework includes commonly used models such as the log-logistic and Weibull models, but also features entire suites of models as found in various guidance documents. An outline on how the proposed framework can be implemented in statistical software systems is also provided. Environ. Toxicol. Chem. 2010;29:220–229.

Keywords—Log-logistic models Logarithm-transformed doses Open source statistical software Parameterizations Weibull models

INTRODUCTION

Dose–response modeling is the state-of-the-art methodology underlying modern risk assessment. As a consequence, a vast number of different models have been proposed for fitting dose–response data and they offer great flexibility. This is a consideration that may be of particular interest for estimation of low effect doses as these are quite sensitive to the choice of the dose–response model [1,2].

However, some aspects of dose–response modeling still appear to confuse ecotoxicologists. A couple of examples may be useful to illustrate this point. First, a wide range of different parameterizations of the models are used. What are the differences and, more essentially, why are different parameterizations used? Second, often logarithm-transformed doses are used. Is it really necessary to use logarithm-transformed doses? Various biological arguments are provided in favor of the logarithm transformation, but they seem to amount to ad hoc procedures applied before the statistical analysis. Can this be correct? Inconsistent, insufficient, or even incorrect naming conventions for the models also cause confusion: What is the difference between the logistic and log-logistic models? How is the Hill model related to the log-logistic models? Is a Weibull model also a Gompertz model? The present study intends to answer these questions. It will be shown that of basic sciences and environment the term Weibull does not guarantee a unique identification of the model used. The main problem with the use of names is that in many studies they are used to completely replace the mathematical definitions of the models, but this requires a one-to-one link between the name of the model and the mathematical equation of the model.

* To whom correspondence may be addressed (ritz@life.ku.dk).

Published online 18 September 2009 in Wiley InterScience (www.interscience.wiley.com).

Therefore, it is highly desirable to establish a unified framework to structure and strengthen the dose–response modeling concepts in use in ecotoxicology. To date, no such unified approach seems to have been proposed, nor have guidelines been developed in national or international contexts that seem to address this issue in any general or conceptual manner [3,4]. The benefits of this approach would be the establishment of a common framework for ecotoxicologists, which enables the use of a more precise terminology regarding dose–response models, eventually making it easier to compare and communicate results.

Thus, the aim of this review is to provide a general framework that helps establish an overview of available dose–response models, to highlight advantages and drawbacks of the different models, and to clarify some issues in the modeling process. The present study demonstrates how a wide range of common and less common dose–response models used in ecotoxicology are interrelated, and to provide recommendations on the usefulness of the different models in an ecotoxicological context.

This review is confined to aspects directly related to modeling the average or systematic dose–response trend in the data. One reason for this constraint is to keep this review focused. Another reason is that a lot of risk assessment still relies solely on the point estimates of parameter of interest such as effective doses, which are derived from the fitted average trend, but uncertainty measures are not always taken into consideration. Moreover, adequate modeling of the average trend is an essential prerequisite to apply procedures for assessing the uncertainty of estimates. It is not the intention of the present study to introduce new dose–response models. The focus is entirely on existing models, because they already provide a very flexible pool of model functions. Consequently, neither estimation procedures (including modifications to adjust for various deviations from the model assumptions, such as transformations and

variance modeling) nor procedures for assessing the uncertainty of estimates of parameter of interest (such as the many ways of obtaining standard errors or confidence interval of effective doses) will be reviewed.

DOSE–RESPONSE MODELS

The systematic dose–response trend of any dose–response model is described by the specific choice of dose–response model function f, which is a function of the dose x. The function f should reflect the average or mean dose–response behavior seen in the data. The choice of f may be based on empirical or theoretical considerations, or, in the absence of such information, data-driven model selection procedures [5,6].

Some studies have focused on dose–response data with continuous endpoints [2,6,7], others on quantal endpoints [8], whereas the general guidelines provided by Environment Canada [3] or the Organization for Economic Co-operation and Development [4] consider models for continuous and quantal dose–response data in separate sections and chapters. In contrast, the present study considers dose–response modeling from a vantage point, encompassing arbitrary endpoints (continuous, count, or quantal endpoints) that could be derived from acute or chronic tests, lethal or sublethal endpoints. This approach is similar to that of Caux and Moore [9].

For continuous and count data, f is describing the size of the effect considered as a function of dose, whereas for quantal data, f is the probability of being affected as a function of dose. This difference may be important for the interpretation of derived parameters such as benchmark doses [6], but is not relevant here as the focus lies on modeling the average trend in the dose–response data. It is customary to assume that the function f is completely known and specified apart from some parameters denoted b, c, d, e,... (where ... is a placeholder for any additional parameter). The parameters are estimated by determining parameter values such that f fits the dose–response data as closely as possible as judged by some criterion, which reflects the distributional assumptions (least squares or maximum likelihood).

The present study considers four different classes of sigmoidal dose–response models with horizontal asymptotes or limits at the extremes of the dose range. It will be shown below that these four classes encompass many of the dose–response models that are useful to ecotoxicologists. The classes are called log-logistic models, log-normal models, Weibull-1 models, and Weibull-2 models. The log-logistic and the log-normal models are symmetric as functions of the logarithm-transformed dose, whereas the Weibull models are asymmetric on the original dose scale. It is worth noting that there are two types of Weibull models, a fact already pointed out by Seber and Wild [10]. Incidentally, the four classes correspond to the four link functions identified for quantal data by McCullagh and Nelder [11]. The models are equally fit for describing decreasing dose–response data as well as increasing dose–effect data. In what follows, decreasing dose–response data (with one exception that will be pointed out) are considered, but the comments will also apply to increasing dose–response data (possibly after appropriate changes of signs in the model functions). More specifically, the present study is about model functions f of the following general form:

$$f(x; b, c, d, e, ...) = c + (d - c)h(x; b, e, ...) \quad (1)$$

for some appropriate choice of the function h of the parameters b, e as well as any additional parameters. The parameters c and d are the lower and upper horizontal asymptotes or limits, respectively, and they are in the same units as the endpoint itself. In other words, d is the average level in the control or untreated group, whereas c is the average level attained at very high doses. The special case with $c = 0$ and $d = 1$ in Equation 1 is often useful for analysis of quantal dose–response data but only rarely for continuous dose–response data, although one example is modeling of optical density as a function of dose in cytotoxic assays [12]. However, in general, rescaling of continuous response values to obtain values between 0 and 1 should be avoided, unless there are very good reasons for applying such a normalization procedure. Alternatively, a nonstandard approach should be applied subsequently to adjust for the effect of rescaling [13]. Rescaling introduces correlation between the scaled response values because the scaling factor is determined from the data (often control data are used to scale data for the remaining doses). Moreover, the rescaling will typically reduce the variation in the data, which will result in too small estimated standard errors for some of the parameter estimates. If rescaling is only applied with a view toward presenting the results (as it appears often to be the case), it should only be applied to the fitted curve after having fitted a dose–response model to the original data.

The sign of the parameter b determines whether the dose–response curve is decreasing or increasing, but, due to the range of models considered, a positive sign will not always correspond to an increasing curve. In any case, a change in sign suggests a change in the monotonicity pattern. Moreover, the parameter b is proportional to the slope of the dose–response curve at the dose e, and typically it assumes a value between 0 and 5, although larger values are also occasionally encountered. As the value of b increases, the dose–response curve becomes steeper. For quantal dose–response data, in the terminology of Finney [14], the steeper the curve, the more concentrated is the underlying tolerance distribution of the test organisms used. For continuous dose–response data, no similar interpretation is available. The parameter e is the inflection point of the dose–response curve for all four classes of models, that is, the point where a change in acceleration in the curve occurs. For the symmetric log-logistic and log-normal models, this parameter also corresponds to the effective dose resulting in a 50% reduction in the response. The inflection point will generally be larger than the median effective dose (ED50) for the Weibull-1 model but smaller for the Weibull-2 model.

As previously mentioned, some dose–response models are not included in the framework of this review. Examples are threshold models such as the hockey stick model [3,15,16] and the hormesis model introduced by Hunt and Bowman [17], but these models are not in common use, which may be due to the lack of a biological rationale for the existence of a threshold. Second-order and higher-order polynomial models, which have occasionally been used for modeling dose–response data [4,18,19] and polynomials in dose within the generalized linear

model framework [20,21], are also not part of the general framework proposed in the present study, but these models are only occasionally useful in a dose–response context, as even interpolation can be unreliable [6]. Some of the more specialized models mentioned by Scholze et al. [2] are also not included.

Log-logistic models

The log-logistic models are by far the most commonly used models for describing dose–response data in toxicology. They occur in a variety of parameterizations, special cases, and extensions throughout the toxicological literature. The four-parameter log-logistic model is defined by the model function:

$$f(x;b,c,d,e) = c + \frac{d-c}{1+\exp(b(\log(x)-\log(e)))} = c + \frac{d-c}{1+\left(\frac{x}{e}\right)^b} \quad (2)$$

The two expressions on the right-hand side are two ways of writing exactly the same model, in the sense that the parameters have the same interpretation and have identical estimates for any dataset considered. A third way of writing exactly the same model as in Equation 2 is by means of logarithms with base 10 rather than the natural logarithm used above. The parameter e is the dose that produces a 50% reduction relative to the control and the lower limit. This is the effective dose ED50. In fact, for quantal data, the median of the underlying tolerance distribution is equal to e, and, therefore, e or ED50 is often referred to as median effective dose [22]. For quantal data involving mortality, e is often denoted LD50 or median lethal dose [23]. In the above parameterization, the four-parameter log-logistic model has been extensively used in the last few decades for modeling toxicity of herbicides [24]. It has also been used for analysis of immunoassay data [25], and more recently in the context of the quantitative polymerase chain reaction technology by Rutledge [26]. It is worth noting that the four-parameter model in Equation 2 may also be appropriate for quantal data in case both natural mortality/response and natural immunity are observed [14]. This comment will also apply to the three other classes of models considered below.

It is desirable to replace some of the parameters with fixed known values whenever biological or other subject-matter considerations entertain such assumptions. One benefit is improved precision of the remaining parameter estimates (as the information content of the model has been enhanced). Another is a simpler estimation problem that may more easily yield meaningful parameter estimates. A common situation, where information is added, is for decreasing dose–response data to fix the lower asymptote c at 0 on biological or toxicological grounds (at high concentrations of an effluent all test organisms die), even though the actual data are not supporting this assumption. The resulting three-parameter model is:

$$f(x;b,d,e) = \frac{d}{1+\exp(b(\log(x)-\log(e)))} \quad (3)$$

This model is used by Stephenson et al. [5]. Rewriting the right-hand side in Equation 3 results in the equation

$$f(x;b,d,e) = \frac{dx^{-b}}{x^{-b}+e^{-b}} \quad (4)$$

The model is often referred to as the Hill equation or model [27,28] and $-b$ is called the Hill slope [4]. From the three-parameter model in Equation 3, two prominent special cases can be derived. First, by letting b be equal to 1 in Equation 3, the decreasing Michaelis-Menten model is obtained:

$$f(x;d,e) = \frac{d}{1+(x/e)} = d - \frac{dx}{x+e} \quad (5)$$

which has been used in ecotoxicology by Meister and van den Brink [18]. The increasing Michaelis-Menten model is obtained in a similar way for $b=-1$ [4].

In a similar way, the shifted Michaelis-Menten model can be obtained from the four-parameter log-logistic model in Equation 2. In passing, it is worth noting that the Michaelis-Menten model can be extended to rational polynomial models involving (fractional) polynomial terms in both numerator and denominator [22,29,30], but the drawbacks are the same as for ordinary polynomial models [6]. The second special case is obtained by fixing the upper limit at 1. The resulting model is:

$$f(x;b,e) = \frac{1}{1+\exp(b(\log(x)-\log(e)))} \quad (6)$$

which is the classic logistic regression model [11], which is a generalized linear model with the logit link [8,20], but with $\log(x)$ (and not x) as the explanatory variable. Thus, for reporting a dose–response analysis, it is not enough simply to state that a logit model was used, even though this seems to be common practice, as the question is whether or not $\log(x)$ was used as an explanatory variable. This comment also applies to the probit model introduced below. A related special case is provided by Caux and Moore [9], who consider the two-parameter model where the upper limit is fixed at 100.

Extensions of the four-parameter log-logistic include the five-parameter or generalized log-logistic model

$$f(x;b,c,d,e,g) = c + \frac{d-c}{(1+\exp(b(\log(x)-\log(e))))^g} \quad (7)$$

which, by virtue of the positive parameter g, results in an asymmetric dose–response curve that has been used in bioassays, immunoassays, and toxicology [31–33]. However, it comes at the cost of one additional parameter, which sometimes makes it somewhat more difficult to fit than the four-parameter model. For $g=1$, the four-parameter log-logistic model is retrieved, whereas $g<1$ pushes the dose–response curve to the right relative to $g=1$ and $g>0$ forces the curve to lie under the curve corresponding to $g=1$. Thus, the kind of asymmetry which this model accommodates corresponds to a more slow/rapid decrease toward the lower limit as compared to the symmetric four-parameter model. The fits of the four- and five-parameter log-logistic models will not differ much near the upper limit (close to the control dose). The generalized log-logistic model appears to have received very little attention among ecotoxicologists under such names as Aranda-Ordaz or generalized logit II [2]. The

generalized logit I model [2], as well as the model proposed by Stukel [9,34], lead to other asymmetric five-parameter extensions of the four-parameter log-logistic model.

Other extensions are dose–response models that describe hormesis, such as the model introduced by Brain and Cousens [35], which includes the simple linear regression model as a special case, a model that is occasionally useful for modeling sublethal toxicity data [3]. It is also worth noting that the Brain-Cousens model is only meaningful for decreasing dose–response data. Another hormesis model derived from the four-parameter log-logistic model is proposed by Cedergreen et al. [36]. Yet other extensions are the general biphasic models introduced by Beckon et al. [37] and the model by Ricketts and Head [38], which is capable of fitting dose–response data with a bent, due to a marked change in slope.

Often the log-logistic model is wrongly termed the logistic model [5,32,39,40], which is defined by the very similar model function

$$f(x; b, c, d, e) = c + \frac{d - c}{1 + \exp(b(x - e))} \quad (8)$$

The main difference from the log-logistic model in Equation 2 is that no logarithm is applied to the terms x and e [41,42]. Replacing x by $\log(x)$ retrieves the log-logistic model, but in a slightly different parameterization where log(ED50) and not ED50 is a parameter in the model.

The logistic model, sometimes referred to as the Boltzmann equation [43], is also a sigmoidal model, but it is defined on the entire real axis, for both positive and nonpositive values of the independent variable x. If both the lower and upper limits of the dose–response relationship are well determined by the data the logistic and log-logistic models produce almost identical fits with the same estimates of c and d. If either the lower or upper limit is poorly determined by the data the logistic model may give estimates of c or d that do not necessarily both have meaningful interpretations in the dose-response context, as they correspond to horizontal asymptotes at the extremes $-\infty$ and ∞, and in such cases the parameter e may be a biased estimate of ED50. This point is illustrated in Figure 1, which shows continuous decreasing dose–response data from a study investigating the inhibitory effect of secalonic acid on plant growth [44]. Four-parameter log-logistic and logistic models were fitted to the data and the fitted dose–response curves are shown in Figure 1; the solid line is the log-logistic model and the long-dashed line is the logistic model. In addition, the horizontal short-dashed line indicates the estimate of the upper asymptote based on the logistic model (for the log-logistic model this estimate is almost identical to the observed control level). It is worth noting that the difference in the estimated upper limits is hardly visible from the fitted curves shown in Figure 1, yet there is an appreciable difference between the estimated upper asymptotes. This data example does not show a dramatic difference between the models, but that could easily be the case in situations with very limited information for the control group. Thus, for dose–response data, the logistic model offers no advantage over the log-logistic model.

For quantal data, this argument may be less compelling, as the lower and upper limits are often, but not always, fixed a priori at 0 and 1 respectively. Extensions of the logistic model similar to the generalized log-logistic model above are called Richards models [10,45].

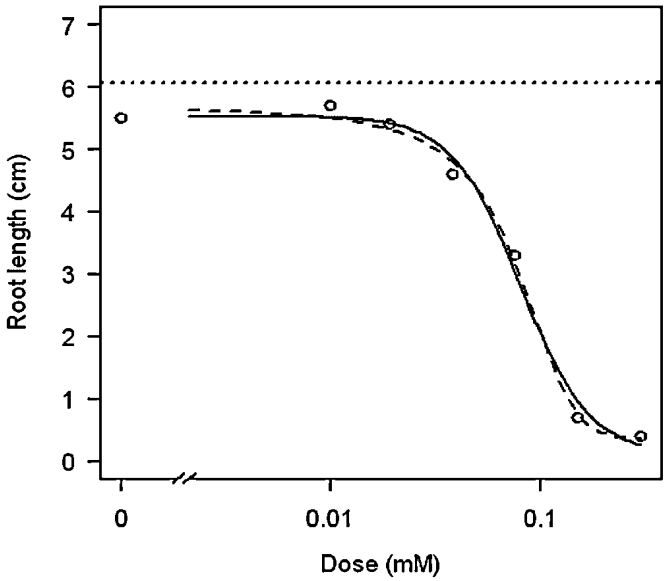

Fig. 1. Comparison of logistic and log-logistic dose–response models for describing reduction in plant growth measured as root length (cm) in response to increasing doses of secalonic acid (mM). Average root lengths are shown. The solid line is the four-parameter log-logistic model with an estimated upper limit of 5.53 (very close to the endpoint value for the control group). The long-dashed line is the four-parameter logistic model with an estimated upper limit of 6.05, which is also indicated (short-dashed horizontal line).

Log-normal models

Bruce and Versteeg [7] introduced the four-parameter log-normal dose–response model. In the present study, the model is defined using a slightly different parameterization in the following way:

$$f(x; b, c, d, e) = c + (d - c)\Phi(b(\log(x) - \log(e))) \quad (9)$$

where Φ is the cumulative distribution function of the standard normal distribution. The special case with the lower limit fixed at 0 is used by van der Hoeven [46], but in practice, this model does not appear to be used very often for continuous endpoints. One reason may be that the general model given in Equation 9 is not readily available in statistical software packages. Moreover, Bruce and Versteeg [7] note that the results from log-normal and log-logistic models are almost identical.

By fixing c and d in Equation 9 at 0 and 1 respectively, the probit model with $\log(x)$ as explanatory variable is obtained. This model is sometimes referred to as the log-probit model [47]. The probit model has been used for a long time for modeling quantal dose–response data [14]. A more recent application in ecotoxicology is found in Wheeler et al. [48]. In many cases, the logit and probit models result in very similar estimates [11]. However, for low effects there may still be some appreciable difference.

Weibull-1 models

The four-parameter Weibull-1 model is defined by the model function

$$f(x; b, c, d, e) = c + (d - c)\exp(-\exp(b(\log(x) - \log(e)))) \quad (10)$$

This is the Weibull growth model considered by Piegorsch and Bailer [22], albeit in a slightly different parameterization. The parameter b is reflecting the steepness of the dose–response curve with large values corresponding to steeper curves, and the parameter e is the dose where the inflection point of the dose–response curve is located. The asymmetry of the Weibull-1 model can be characterized as follows: the dose–response curve descends slowly from the upper limit, but on the other side, the curve approaches the lower limit rapidly. Some graphical illustrations of the two types of Weibull models are provided below. In fact, at the upper limit the Weibull-1 model is very similar to the log-logistic model (which can be seen by using the approximation $\exp(u) \approx 1 + u$ for μ close to 0). Thus, if low toxic effects are of interest, as they often are in toxicology, there is no need to consider both the log-logistic and Weibull-1 models (for decreasing dose–response data) as they produce almost identical results from the two models (tacitly assuming that both models attain roughly the same lower asymptote for high doses, as is very often the case in practice).

The three-parameter Weibull-1 model obtained by fixing the lower limit (c) at 0 is described in more detail in Environment Canada [3], but under the name of Gompertz. The same naming inconsistency also appears in Sand et al. [33] and Cedergreen et al. [36]. In fact, there seems to be a general confusion about names for Weibull models. For example, Scholze et al. [2] claim that the Weibull-2 model introduced below is also called a Gompertz model. This is also not the correct name. Some of the confusion may derive from the fact that a Gompertz model with $\log(x)$ (and not x) is a Weibull-1 model. In other words, the basic difference between the Gompertz and the Weibull model is the form of the exponent: a Gompertz model is the exponential of an exponential in *dose*: $\exp(b\exp(x))$ [22]. The Weibull model, on the other hand, is an exponential of a power in *dose*: $\exp(x^b)$ [22]. One implication of this distinction is that the Gompertz model, in principle, is defined for the entire real axis and therefore the same comments apply as for the logistic model (modifications exist that give increasing growth curves defined on the nonnegative axis only [28]). In conclusion, the Gompertz model as a model in dose is not really useful for modeling dose–response data.

The exponential model with a non-zero lower limit is defined by the model function

$$f(x; c, d, e) = c + (d - c)\exp(-x/e) \quad (11)$$

This model is obtained by fixing b at 1 in Equation 10. The exponential model with lower limit at 0 ($c = 0$) is used by Stephenson et al. [5] (in a slightly different parameterization) for analysis of soil toxicity test data.

All models described by Slob [6] are Weibull-1 models in the sense that they can be derived from the Weibull-1 model in Equation 10 or some special case obtained by fixing one or more parameters at specific values, possibly in a different parameterization.

The special case of the Weibull-1 model resulting from fixing $c = 0$ and $d = 1$ could be useful for quantal dose–response data, but this model appears not to have been used in practice by ecotoxicologists. This is not entirely surprising, as the corresponding generalized linear model with log-log link function for quantal data is also rarely used by statisticians.

Weibull-2 models

The second class of Weibull models is defined by the model function:

$$f(x; b, c, d, e) = c + (d - c)(1 - \exp(-\exp(b(\log(x) - \log(e))))) \quad (12)$$

This model is different from theWeibull-1 model in that it exhibits a different form of asymmetry with a rapid change or descent from the upper limit, but a slow approach toward the lower limit. Thus, the Weibull-2 model is different from the log-logistic model close to the upper asymptote (low doses for decreasing data), but very similar to the log-logistic close to the lower asymptote. The Weibull-2 model has been used in ecotoxicology by Scholze et al. [2], among others.

Figure 2 shows decreasing continuous dose–response data from a study on root growth inhibition of perennial ryegrass being treated with phenolic acid [49]. Average root lengths are shown for each dose. Three dose–response models were fitted and they are also shown in Figure 2: the four-parameter log-logistic (solid line), Weibull-1 (long-dashed line), and Weibull-2 (short-dashed line) models. For low effects, the log-logistic and Weibull-1 models are almost indistinguishable, whereas the Weibull-2 model exhibits a sharper ascent toward the upper

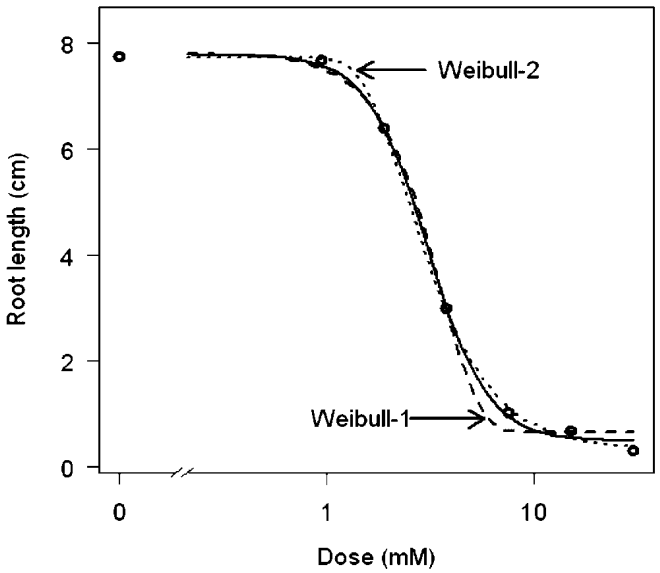

Fig. 2. Comparison of log-logistic, Weibull-1, and Weibull-2 models for decreasing continuous dose–response data from an experiment where perennial ryegrass was exposed to increasing doses of phenolic acid (mM). The endpoint measured is root length (cm). The solid line is the four-parameter log-logistic model. The long-dashed line is the Weibull-1 model and the short-dashed line is the Weibull-2 model.

asymptote. For high effects, the picture is reversed: The log-logistic and Weibull-2 models are very similar, but the Weibull-1 model deviates by more rapidly reaching the lower asymptote. In the middle region, between the asymptotes, the three models provide very identical fitted curves.

The special case with $c = 0$ is sometimes called the Douglas model in pharmacology [28]. Caux and Moore [9] consider the case with the constraints $c = 0$ and $d = 100$.

Another model that deserves mention, as it is related to the multistage models [22,50] and the Hodgkin model in pharmacology [28], is obtained by setting $b = 1$:

$$f(x; c, d, e) = c + (d - c)(1 - \exp(-x/e)) \qquad (13)$$

This model is the increasing asymptotic regression model that has been used to describe weight increase as function of supplementation dose levels [51]. It is an asymmetric alternative to the increasing Michaelis-Menten model. In fact, by fixing d at 1 in Equation 13, the first-order multistage model implemented in the Benchmark Dose Software program (developed by the U.S. Environmental Protection Agency [U.S. EPA] and available on their website [http://www.epa.gov/NCEA/bmds]) is obtained (in a slightly different parameterization than what is used in BMDS). Figure 3 shows increasing quantal dose–response data from a cancer bioassay used in the online tutorial of the BMDS software (Example 2 in IV. Application of BMDS). The first-order multistage model, which is a type of Weibull-2 model, as well as the corresponding log-logistic and Weibull-1 models, were fitted to the data. All three models contain 2 parameters: c and e. The fitted dose–response curves are also shown in Figure 3. The log-logistic and multistage models provide very similar fits, but the Weibull-1 model results in a different but improved fit, as was to be expected around the lower asymptote. For quantal data, the special case

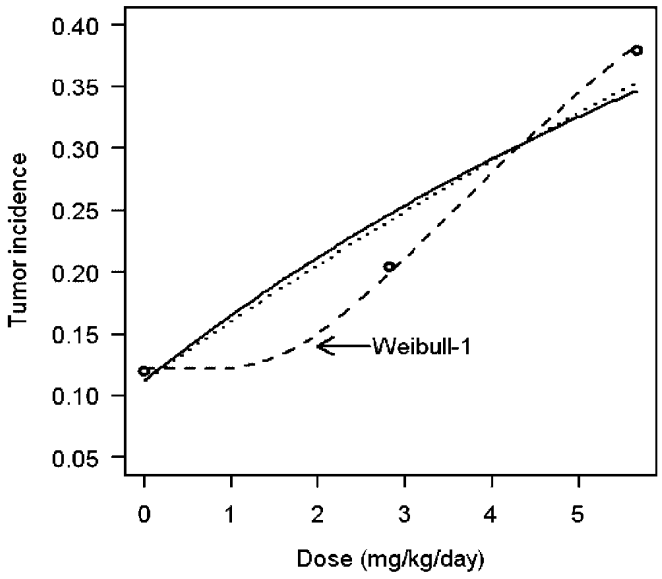

Fig. 3. Comparison of log-logistic, Weibull-1, and Weibull-2 models for increasing quantal dose–response data showing tumor incidence as a function of increasing doses of a carcinogen (mg/kg/d). The solid line is the two-parameter log-logistic model. The long-dashed line is the Weibull-1 model and the short-dashed line is the Weibull-2 model.

with $c = 0$ and $d = 1$ is useful [18]. In another parameterization, this special case is the well-known generalized linear model with the complementary log-log link function for quantal data [11,22], and it is available through most statistical software packages. Finally, the special case obtained by fixing the three parameters c, d, and e at the values 0, 1, and 1, respectively, is the one-hit model described by Edler and Kopp-Schneider [52].

The models discussed in this section are summarized in Table 1.

USE OF DIFFERENT PARAMETERIZATIONS

The systematic trend described by a dose–response model is determined by the specific choice of dose–response function f. The specification can be in terms of a name, but this requires that it is generally agreed upon which dose–response model function f is being referred to (naming conventions). This study seems to indicate that such a general understanding has not yet been broadly established in ecotoxicology. The proposed framework in the previous section could promote such a general understanding. Alternatively, the equation of the dose–response function can be specified explicitly by providing the corresponding equation. This specification is also in common use and avoids ambiguity.

However, there is one complication. There are many ways of mathematically specifying the same dose–response model function, often referred to as different parameterizations. To replace one parameterization by another parameterization is often termed to re-parameterize. A simple dose–response model may serve as example to illustrate how to think about different parameterizations. The exponential model $d\exp(-x/e)$ with $e > 0$, which is a special case of Equation 11 for $c = 0$, is useful for modeling decreasing dose–response patterns that only exhibit asymptotic behavior as the dose x becomes very large. Another parameterization of the same model is $d\exp(-\tilde{e}x)$. The relationship between the two parameters e and $\tilde{e}$ is $e = 1/\tilde{e}$ (the parameter d has the same interpretation in both parameterizations). Yet another parameterization of the same model would be $d\exp(-x/(\tilde{e}^2))$ with the following relationship to the original parameter $\tilde{e} = \sqrt{e}$. So there will almost always be several parameterizations of the same model. Moreover, it will always be the case that for different parameterizations of same model, the parameters in one parameterization can be obtained from the parameters in any other parameterization through mathematical manipulations.

Often different parameterizations of the same model are used to estimate different parameters of interest. Several studies have pursued this endeavor and succeeded in deriving parameterizations suitable for estimation of various parameters of interest [5,41,53,54]. Thus for each parameter of interest, another parameterization was fitted to the same dose–response data. It may seem a convenient way to estimate parameters of interest, but in practice this approach may lead to some undesirable side effects, such as the negative lower bounds for confidence intervals of ED20 as found in Stephenson et al. [5].

Therefore, the usefulness of re-parameterizations is limited. It should suffice to use a single parameterization to estimate all parameters of interest together with the corresponding measures of uncertainty. One problem with the use of several parameterizations is that different parameterizations may behave very

Table 1. Model functions and special cases for the four classes of dose–response models[a]

Model	Model function/Equation	Special cases
log-logistic	$c + \frac{d-c}{1+\exp(b(\log(x)-\log(e)))} = c + \frac{d-c}{1+\left(\frac{x}{e}\right)^b}$	Log-logit (logistic regression), Hill, Michaelis-Menten
log-normal	$c + (d-c)\Phi(b(\log(x)-\log(e))$ (Φ is the cumulative distribution function of the standard normal distribution)	Log-probit
Weibull-1	$c + (d-c)\exp(-\exp(b(\log(x)-\log(e))))$	Exponential, log-log link
Weibull-2	$c + (d-c)(1-\exp(-\exp(b(\log(x)-\log(e)))))$	Complementary log-log link, first-order multistage, one-hit

The parameters c and d denote the horizontal asymptotes or limits, whereas the parameter b is the relative slope at the inflection point of the resulting dose–response curve. The parameter e corresponds to the dose where the inflection point is located. The original, untransformed dose is denoted x throughout.

differently during the estimation process. Some parameterizations are quite robust and will, in most cases, lead to useful parameter estimates, whereas for other parameterizations, such as those used to estimate low effect doses, it can be very difficult to obtain reasonable estimates. Re-parameterization is also not a viable solution for practitioners or for automated dose–response analysis of high-throughput data. Thus, the present study encourages the use of a single, robust parameterization combined with subsequent after-fitting to derive any estimates of parameter of interest with accompanying measures of uncertainty. Thus, the dose–response model that is actually fitted is simply fitted because it is the most convenient model to use for estimation. The parameterizations used in the present study may be a good place to start. At least some of the models have been extensively tested through the availability in an open-source environment [55]. Moreover, from a statistical software perspective, different parameterizations were only needed due to limitations in the capabilities of available statistical software. With the advent of more dynamic and powerful statistical software in the last decade, there is no longer a need to find ways to circumvent these limitations. This development will eventually lead to more transparent estimation procedures.

Occasionally, it may be necessary to decide in advance on which parameterization to use. This has to do with the sample size and the parameters of interest. Typically, it is assumed that parameter estimates are approximately normally distributed, and in this case, the corresponding asymptotically based standard errors are obtained using a local linear approximation to the objective function derived from the criterion used to obtain estimates (e.g., maximum likelihood or least squares). For small dose–response datasets (size of the total dose–response dataset < 20–30), the normality assumption may be questionable and, as a consequence, the obtained standard errors may be inadequate or even misleading measures of the uncertainty or variation in the distribution of the estimate. Choosing another parameterization may alleviate this problem in some cases. By definition, the parameter e is often located somewhere in the middle part of dose range considered. This fact is convenient for interpretation, but in some cases less so for estimation of the uncertainty associated with e. The parameter e needs to be positive, and, therefore, the distribution of the estimate of e may be right skewed; the smaller the dataset. the more pronounced is the skewness. as the distribution will be less concentrated, and hence it may be problematic to approximate the distribution by a normal distribution in such cases. If instead dose–response models that have the parameter e replaced by $\exp(\tilde{e})$ ($\tilde{e}$ denotes a new parameter with values on the entire real axis) are considered, it may often be more reasonable to assume that the estimate of $\tilde{e}$ is approximately normally distributed, which in turn allows the use of standard asymptotically based results to obtain standard errors and tests. Subsequently, the estimated parameters, as well as the corresponding confidence intervals, can be back-transformed to the original dose scale (this ensures positive estimates and lower bounds on confidence intervals). This approach has mainly been used for ED50 in log-logistic and log-normal models [48,56]. Similarly, a parameterization in terms of the parameters b and $\tilde{e}$ will also provide a means of calculating estimates of logarithm-transformed ED values, which subsequently can be back-transformed to the original dose scale.

LOGARITHMIC DOSES

It is a common misperception among biologists and ecotoxicologists that the doses should be logarithm-transformed *before* they enter the statistical dose–response analysis.

This approach has recently been enforced by Environment Canada [3], based on the biological rationale that the test organisms experience the dose stimulus on a multiplicative scale rather than an additive scale. Similarly, for probit analysis, Finney [14] argues that the underlying distribution of tolerance doses in the population of test organisms is concentrated on the non-negative dose axis and is right skewed. Consequently, he argues that a logarithm transformation often helps to obtain a symmetric distribution close to a normal distribution that would justify the probit analysis.

More important than speculating about unobservable tolerance distributions, the logarithm transformation ensures that the resulting probit model is based on a reasonable dose–response model: The horizontal asymptotes are located at the extremes 0 and ∞ of the dose range, which is very reasonable for dose–response data, and not at $-\infty$ and ∞ as would be the case if the logarithm transformation had not been applied. In fact, this is the profound consequence of using the logarithm-transformed doses. Whether or not the unobserved tolerance distribution is normal is another question, which is often very difficult to answer in practice. Frequently this may not be the case, and this was, in part, the motivation for introducing the broad family of models termed generalized linear models for quantal data [11], which are very flexible regression models that include the probit model as one of several models suitable for quantal data. The logit model and the two-parameter Weibull models ($c = 0$ and $d = 1$) are other choices.

Thus, the use of logarithm-transformed doses is a means for obtaining dose–response models that are defined on the nonnegative dose axis with certain desirable, biologically

meaningful properties (s-shape with horizontal asymptotes at the control and at very large doses). However, this reasoning need not hold for all potential dose–response models. A more appropriate approach (than adhering blindly to the use of the logarithm transformation) is to define dose–response models that explicitly possess meaningful properties and thereby reflect biological constraints.

In many cases, the biological explanations are very plausible. However, the manner in which these explanations are used is problematic in the subsequent analysis of the data, as they seem to lead to ad hoc adjustments and transformations of the doses before the statistical dose–response analysis, not clearly separating biological considerations from data manipulation. In sharp contrast to this, the present study encourages the use of dose–response models that are formulated in terms of the original dose (and not logarithm-transformed dose or any other type of transformed dose) and that directly and transparently incorporate any available biological information.

For example, the models within the proposed framework are biologically meaningful as they describe typical dose–response patterns, and at the same time, they are mathematically well-defined, continuous, and smooth for positive doses as well as the control at dose 0, even though the control often has been handled as a special case in statistical software programs. A closer look will reveal that exponentials and logarithms annul each other in an appropriate sense and in reality there is no paradox. This approach also avoids the common subterfuge of having to apply ad hoc adjustments, such as adding a small positive value to all doses to shift the control dose slightly away from 0. These ad hoc adjustments are yet another undesirable consequence of applying the logarithm transformation before the statistical analysis.

On a related note, it is worth mentioning that the use of logarithmic dose series, as suggested by Environment Canada [3], is not required for using a dose–response model. Sometimes it may still be convenient and practical to use logarithmic dose series for some types of dose–response experiments, but it is not a universal requirement. In fact, it could be seen as yet another remnant more suitable for analysis of variance models than for regression models. Moreover, there is an increasing understanding that more doses and less replicates are needed to exploit the regression approach fully [5]. For capturing hormetic effects, logarithmic doses may sometimes be useful, but often the dose range where hormesis occurs is so narrow that doses have to be determined using some other dose scheme, such as equidistantly spaced doses [57].

Once a dose–response model has been fitted, the estimated parameter and the fitted dose–response curve can be interpreted, reproduced, and transformed in whatever way that is biologically meaningful in a given context, such as relative potencies used for comparisons of toxicity [58,59]. The key point is that all these manipulations should only be done *after* fitting the dose–response model.

SOFTWARE CONSIDERATIONS

There have been several initiatives to provide easily accessible software for dose–response modeling to ecotoxicologists through spreadsheet-based add-ons [9,27,60]. Several stand-alone programs have also been developed over the last decades, such as the programs developed by Kalliomaa et al. [16], but they seem to have vanished again. Another freely available program is the U.S. EPA's Benchmark Dose Software, specifically designed toward benchmark dose estimation. Moreover, several commercial programs, both specialized tools for dose–response modeling in toxicology and multi-purpose statistical software, are commonly used in ecotoxicology; some of these programs are mentioned in Environment Canada [3], Organization for Economic Cooperation and Development [4], Caux and Moore [9], and Motulsky and Christopoulos [56]. It appears that none of these specialized programs covers all the dose–response models proposed in the present study. In principle, the multi-purpose programs can be used for fitting any dose–response model, but they may be difficult for nonstatisticians to use. Another issue is that most programs will make a distinction between continuous, count, quantal, and perhaps even event times, possibly requiring quite different interfaces or specifications, and, thus, no unified view on dose-response modeling is entertained.

The natural hierarchical structure between models within each of the four classes in the proposed framework is very convenient for general software implementation, as models that are special cases inherit most features from the parent model. Currently, this approach is being implemented through the extension package drc [55] for the open source statistical software R, which is developed by the R Project for Statistical Computing (http://www.r-project.org) [61]. This software provides a very flexible platform for implementing such hierarchical structures. However, it may be possible to implement similar structures within other statistical software packages and programs, but it will be highly dependent on the underlying infrastructure of the software.

Apart from the advantages pertaining to the implementation, the open source software development also satisfies some of the requirements that have been voiced previously in ecotoxicology [9]: The software is adaptable, as the source code is freely available and can be modified by anyone (with a basic programming understanding) in whatever way needed to suit specific requirements (such as suitability in a given industrial or regulatory context). A related advantage is that the open source development is transparent and any implemented procedurecan be checked and validated directly by inspection of the source code, and not just by applying the software to some suite of test data (the source code in the package drc was through peer-review [55]). The open-source nature of the project also encourages users to actively contribute to the improvement of the software. Extendibility has turned out to be the most powerful feature of R and much functionality, which is currently available in R, is provided by extension packages such as the above-mentioned drc. In this way, it is possible to create new extensions that rely on previously developed extensions and thus to build on top of the existing infrastructure.

The current implementation in R allows convenient specification and fitting of arbitrary special cases that can be obtained by fixing one or more of the model parameters in the models within the proposed framework. The most prominent special cases, however, are built in. Moreover, the software features automatic calculation of starting values, which is an improvement over the manual methods used in the past, but also

described recently [22,56], lack-of-fit tests are available, simultaneous fitting of several curves [39], estimation and comparison of arbitrary ED levels, and convenient plotting functionality. In short, drc and R offer an extremely versatile toolbox for dose–response modeling based on concepts that comply with requirements put forward in industrial, regulatory, and research contexts [3,9]. The dose–response analyses shown in Figures 1, 2, and 3 were produced using drc and R.

CONCLUSION

The present study provides a unified framework that covers most dose–response models of practical interest to ecotoxicologists. For instance, the framework encompasses the suite of dose–response models proposed by Slob [6], as well as all models suggested by Environment Canada [3] (except the hockey stick model) and Organization for Economic Co-operation and Development [4]. Moreover, the present study has endeavored to clarify issues related to the use of different parameterizations and the use of logarithmic doses in dose–response modeling. The present study also highlights some of the key points related to an open source implementation of the proposed framework.

The recommendations of this review can be summarized as follows: the present study recommends avoiding preprocessing of doses (such as logarithm-transforming doses) and normalization of response values before the statistical analysis. If applied, preprocessing or normalization procedures should be described in detail. The dose–response model used in the statistical analysis should be described either by means of the mathematical equation for the model function or by a reference to the specific equation used. It should be ensured that the statistical model makes due allowance for the type of dose–response data (quantal/continuous).

Acknowledgement—The author is greatly indebted to Jens C. Streibig, who has been an enlightening, enthusiastic, and inspiring advocate of dose–response methodology.

REFERENCES

1. Moore DRJ, Caux PY. 1997. Estimating low toxic effects. *Environ Toxicol Chem* 16:794–801.
2. Scholze M, Boedeker W, Faust M, Backhaus T, Altenburger R, Grimme LH. 2001. A general best-fit method for concentration-response curves and the estimation of low-effect concentrations. *Environ Toxicol Chem* 20:448–457.
3. Environment Canada 2005. Guidance document on statistical methods for environmental toxicity tests. Report EPS 1/RM/46. Ottawa, ON.
4. Organization for Economic Cooperation and Development 2006. Current Approaches in the Statistical Analysis of Ecotoxicity Data: A Guidance to Application. Chapter 6. Paris, France, pp 62–102.
5. Stephenson GL, Koper N, Atkinson GF, Solomon KR, Scroggins RP. 2000. Use of nonlinear regression techniques for describing concentration-response relationships of plant species exposed to contaminated site soils. *Environ Toxicol Chem* 19:2968–2981.
6. Slob W. 2002. Dose-response modeling of continuous endpoints. *Toxicol Sci* 66:298–312.
7. Bruce RD, Versteeg DJ. 1992. A statistical procedure for modeling continuous toxicity data. *Environ Toxicol Chem* 11:1485–1494.
8. Kerr DR, Meador JP. 1996. Modeling dose response using generalized linear models. *Environ Toxicol Chem* 15:395–401.
9. Caux PY, Moore DRJ. 1997. A spreadsheet program for estimating low toxic effects. *Environ Toxicol Chem* 16:802–806.
10. Seber GAF, Wild CJ. 1989. *Nonlinear Regression*. John Wiley, New York, NY, USA.
11. McCullagh P, Nelder JA. 1989. *Generalized Linear Models*, 2nd ed. Chapman & Hall, Boca Raton, FL, USA.
12. Baharith LA, Al-Khouli A, Raab GM. 2006. Cytotoxic assays for screening anticancer agents. *Stat Med* 25:2323–2339.
13. Strodl Andersen J, Holst H, Spliid H, Andersen H, Baun A, Nyholm N. 1998. Continuous ecotoxicological data evaluated relative to a control response. *J Agric Biol Environ Stat* 3:405–420.
14. Finney DJ. 1971. *Probit Analysis*, 3rd ed. Cambridge University, London, UK.
15. Klepper O, Bedaux JJM. 1997. Nonlinear parameter estimation for toxicological threshold models. *Ecol Model* 102:315–324.
16. Kalliomaa K, Haag-Grönlund M, Victorin K. 1998. A new model function for continuous data sets in health risk assessment of chemicals using the benchmark dose concepts. *Regul Toxicol Pharmacol* 27:98–107.
17. Hunt DL, Bowman D. 2004. A parametric model for detecting hormetic effects in developmental toxicity studies. *Risk Anal* 24:65–72.
18. Meister R, van den Brink, PJ. 2000. The analysis of laboratory toxicity experiments. In Sparks T, ed, *Statistics in Ecotoxicology*. John Wiley, Chichester, UK, pp 99–118.
19. Gaylor DW, Lutz WK, Conolly RB. 2004. Statistical analysis of nonmonotonic dose-response relationships: Research design and analysis of nasal cell proliferation in rats exposed to formaldehyde. *Toxicol Sci* 77:158–164.
20. Bailer AJ, Oris JT. 1997. Estimating inhibition concentrations for different response scales using generalized linear models *Environ Toxicol Chem* 16:1554–1559.
21. Bailer AJ, Hughes MR, Denton DL, Oris JT. 2000. An empirical comparison of effective concentration estimators for evaluating aquatic toxicity test responses. *Environ Toxicol Chem* 19:141–150.
22. Piegorsch WW, Bailer AJ. 2005. *Analyzing Environmental Data*. John Wiley, Chichester, UK.
23. Williams DA. 1986. Interval estimation of the median lethal dose. *Biometrics* 42:641–645.
24. Streibig JC, Rudemo M, Jensen JE. 1993. Dose-response curves and statistical models. In Streibig JC, Kudsk P, eds, *Herbicide Bioassays*. CRC, Boca Raton, FL, USA, pp 29–55.
25. Dudley RA, Edwards P, Ekins RP, Finney DJ, McKenzie IGM, Raab GM, Rodbard D, Rodgers RPC. 1985. Guidelines for immunoassay data processing. *Clin Chem* 31:1264–1271.
26. Rutledge RG. 2004. Sigmoidal curve-fitting redefines quantitative real-time PCR with the prospective of developing automated high-throughput applications. *Nucleic Acids Res* 32:e178.
27. Vindimian E, Robaut C, Fillion G. 1983. A method for cooperative and noncooperative binding studies using nonlinear regression analysis on a microcomputer. *J Appl Biochem* 5:261–268.
28. Keller F, Giehl M, Czock D, Zellner D. 2002. PK-PD curve-fitting problems with the Hill equation? Try one of the 1-exp functions derived from Hodgkin, Douglas or Gompertz. *Int J Clin Pharmacol Ther* 40:23–29.
29. Kelman GR. 1966. Digital computer subroutine for the conversion of oxygen tension into saturation. *J Appl Physiol* 21:1375–1376.
30. López S, France J, Gerrits WJJ, Dhanoa MS, Humphries DJ, Dijkstra J. 2000. A generalized Michaelis-Menten equation for the analysis of growth. *J Anim Sci* 78:1816–1828.
31. Finney DJ. 1979. Bioassay and the practice of statistical inference. *Int Stat Rev* 47:1–12.
32. Gottschalk PG, Dunn JR. 2005. The five-parameter logistic: A characterization and comparison with the four-parameter logistic. *Anal Biochem* 343:54–65.
33. Sand S, von Rosen D, Victorin K, Falk Filipsson A. 2006. Identification of a critical dose level for risk assessment: Developments in benchmark dose analysis of continuous endpoints. *Toxicol Sci* 90:241–251.
34. Stukel TA. 1988. Generalized logistic models. *J Am Stat Assoc* 83:426–431.
35. Brain P, Cousens R. 1989. An equation to describe dose responses where there is stimulation of growth at low dose. *Weed Res* 29:93–96.
36. Cedergreen N, Ritz C, Streibig JC. 2005. Improved empirical models describing hormesis. *Environ Toxicol Chem* 24:3166–3172.
37. Beckon WN, Parkins C, Maximovich A, Beckon AV. 2008. A general approach to modeling biphasic relationships. *Environ Sci Technol* 42:1308–1314.
38. Ricketts JH, Head GA. 1999. A five-parameter logistic equation for investigating asymmetry of curvature in baroreflex studies. *Am J Physiol Regul Integr Comp Physiol* 277:441–454.

39. De Lean A, Munson PJ, Rodbard D. 1978. Simultaneous analysis of families of sigmoidal curves: Application to bioassay, radioligand assay, and physiological dose-response curves. *Am J Physiol* 235:E97–E102.
40. MacDougall J. 2006. Analysis of dose-response studies: Emax model. In Ting N, ed, *Dose Finding in Drug Development*. Springer, New York, NY, USA, pp 127–145.
41. Zwietering MH, Jongenburger I, Rombouts FM, van'T Riet K. 1990. Modeling of the bacterial growth curve. *Appl Environ Microbiol* 56:1875–1881.
42. Venegas JG, Harris RS, Simon BA. 1998. A comprehensive equation for the pulmonary pressure-volume curve. *J Appl Physiol* 84:389–395.
43. Brown AM. 2001. Nonlinear regression analysis of data using a spreadsheet. *Am Biotechnol Lab* 19:58–60.
44. Gong X, Zeng R, Luo S, Yong C, Zheng Q. 2004. Two new secalonic acids from Aspergillus Japonicus and their allelopathic effects on higher plants. *Proceedings*, International Symposium on Allelopathy Research and Application, Shanshui, Guangdong, China, April 27–29, pp 209–217.
45. Bates DM, Watts DG. 1988. *Nonlinear Regression Analysis and Its Applications*. John Wiley, New York, NY, USA.
46. van der Hoeven N. 1997. How to measure no effect. Part III: Statistical aspects of NOEC, ECx and NEC estimates. *Environmetrics* 8:255–261.
47. Sand S, Victorin K, Falk Filipsson A. 2008. The current state of knowledge on the use of the benchmark dose concept in risk assessment. *J Appl Toxicol* 28:405–421.
48. Wheeler MW, Park RM, Bailer AJ. 2006. Comparing median lethal concentration values using confidence interval overlap or ratio tests. *Environ Toxicol Chem* 25:1441–1444.
49. Inderjit Streibig JC, Olofsdotter M. 2002. Joint action of phenolic acid mixtures and its significance in allelopathy research. *Physiol Plant* 114:422–428.
50. Downs T. 1992. Biostatistical approaches for modeling U-Shaped dose-response curves and study design considerations in assessing the biological effects of low doses. In Calabrese EJ, ed, *Biological Effects of Low Level Exposures to Chemicals and Radiation*. Lewis, Boca Raton, FL, USA, pp 119–145.
51. Kratzer DD, Littell RC. 2004. Appropriate statistical methods to compare dose responses of methionine sources. *Poult Sci* 85:947–954.
52. Edler L, Kopp-Schneider A. 1998. Statistical models for low dose exposure. *Mutat Res* 405:227–236.
53. van Ewijk PH, Hoekstra JA. 1993. Calculation of the EC50 and its confidence interval when subtoxic stimulus is present. *Ecotoxicol Environ Saf* 25:25–32.
54. Schabenberger O, Tharp BE, Kells JJ, Penner D. 1999. Statistical tests for hormesis and effective dosages in herbicide dose response. *Agron J* 91:713–721.
55. Ritz C, Streibig JC. 2005. Bioassay analysis using R. *J Stat Softw* 12:1–22.
56. Motulsky HJ, Christopoulos A. 2004. *Fitting Models to Biological Data Using Linear and Nonlinear Regression: A Practical Guide to Curve Fitting*. Oxford University, Oxford, UK.
57. Belz RG. 2008. Stimulation versus inhibition: Bioactivity of parthenin, a phytochemical from Parthenium hysterophorus L. *Dose Response* 6:80–96.
58. Oris JT, Bailer AJ. 1997. Equivalence of concentration-response relationships in aquatic toxicology studies: testing and implications for potency estimation. *Environ Toxicol Chem* 15:2204–2209.
59. Ritz C, Cedergreen N, Jensen JE, Streibig JC. 2006. Relative potency in nonsimilar dose-response curves. *Weed Sci* 54:407–412.
60. Onofri A. 2005. BIOASSAY97: A new Excel VBA macro to perform statistical analyses on herbicide dose-response data. *Riv Ital Agrometeorol* 3:40–45.
61. R Development Core Team 2009. R: A language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.